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AB Endothelial cell migration was studied following a mechanical injury produced in cultured confluent monolayers of calf aortic endothelium with the use of a quantitative migration **assay**. In this method the cells were grown on glass coverslips coated with scarlet red-containing Formvar. At confluency, the cultures were cut in half with a blade; one half was removed with the pigmented Formvar, and the other was returned to culture. Migration was linear for a least 96 hours, and was due to cell motility, not proliferation. Since it was blocked in the presence of L-azetidine carboxylic acid or **cis-hydroxyproline**, inhibitors of collagen secretion, endothelial cell migration appeared to be dependent on the continual secretion of collagen. Furthermore, the types, apparent relative amounts, and localizations of the collagens as well as laminin changed during the migratory process. These studies support the notion that the aortic endothelial cell migratory response to injury is a dynamic one requiring the continual secretion and modulation of matrix molecules.

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Aortic Endothelial Cell Migration

I. Matrix Requirements and Composition

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Endothelial cell migration was studied following a mechanical injury produced in cultured confluent monolayers of calf aortic endothelium with the use of a quantitative migration assay. In this method the cells were grown on glass coverslips coated with scarlet red-containing Formvar. At confluency, the cultures were cut in half with a blade; one half was removed with the pigmented Formvar, and the other was returned to culture. Migration was linear for at least 96 hours, and was due to cell motility, not proliferation. Since it was blocked in the presence of L-azetidine carboxylic acid or *cis*-hydroxyproline, inhibitors of collagen secretion, endothelial cell migration appeared to be dependent on the continual secretion of collagen. Furthermore, the types, apparent relative amounts, and localizations of the collagens as well as laminin changed during the migratory process. These studies support the notion that the aortic endothelial cell migratory response to injury is a dynamic one requiring the continual secretion and modulation of matrix molecules. (Am J Pathol 1982, 106:180–186)

THE MIGRATORY RESPONSE occurring as a result of vascular injury appears to be a general phenomenon, because it has been observed in various vascular beds *in vivo* and *in vitro* with the use of a variety of techniques.[1-5] In large vessels following injury endothelial migration reconstitutes a flat, continuous, nonthrombogenic surface lining the vessel lumen. In contrast, in capillaries following soft tissue injury, or in response to tumor or inflammatory factors, there is neovascularization by capillary ingrowth; ie, endothelial cells undergo budding and form new lumens. In addition, the process of migration, as observed *in vitro*, is noticeably different for large vessel and capillary-derived endothelial cells. Arterial (aortic) endothelial cell migration is apparently a constitutive function in culture, while capillary endothelial cell migration appears to be an inducible phenomenon triggered by heparin or tumor-conditioned medium that requires a gelatin substratum.[6,7] In light of the close physical association of endothelial cells with the underlying matrix *in vivo* and the matrix requirements noted for capillary endothelial cell migration *in vitro*,[8] an investigation into the interactions of the large vessel endothelial cells with matrix components during the migratory response is warranted. In this study the matrix molecules associated with migrating and stationary aortic endothelial cells were compared with the use of affinity-purified antibodies. The sensi-

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tivity of endothelial cell migration to the known inhibitors of collagen secretion, L-azetidine carboxylic acid and *cis*-hydroxyproline, was also studied.

Materials and Methods

Cover Glasses

Endothelial cell migration was assessed with the use of a technique previously described.[9] Briefly, cover glasses (22 × 22 mm, No. 1, Scientific Products, Division of American Hospital Supply Corp., McGraw Park, Ill) that had been washed sequentially in concentrated sulfuric acid, tap water, distilled water, and absolute ethanol and air-dried, were dipped in ethylene dichloride (Ladd Research Industries, Burlington, Vt) containing 1% Formvar powder (15/95 Grade, Ladd Research Industries, Burlington, Vt) and 0.2% scarlet red (Sudan IV, Allied Chemical Corp., New York, NY). The coverslips were air-dried on sterile paper towels and placed in 35 × 10-mm tissue culture dishes (Falcon Plastics, Oxnard, Calif).

Endothelial Cell Cultures

Bovine (calf) aortic endothelial cells were isolated, cultured, and characterized as previously described.[10] Briefly, endothelial cells were isolated by collagenase treatment, cultured, and passaged at confluency. All experiments were performed between the second and tenth passage. Cells were identified as endothelial cells by anti-factor VIII fluorescence, characteristic structure, and the presence of 5'nucleotidase and angiotensin-converting enzyme activities.[10]

Migration Studies

Each coverslip/dish was inoculated with 10^5 cells, placed in a 37 C humidified atmosphere containing 5% CO_2, 95% air, until confluency was achieved. After irradiation (3000 rads X-ray, Siemens Stabilipan 250; no added filter) to arrest proliferation, the coverslips were removed and placed in sterile Petri dishes (100 mm), and half the Formvar sheet was cut away with a sterile razor blade.[9] The coverslips were then placed in fresh medium (199 E containing 20% heat-inactivated fetal calf serum [GIBCO], penicillin [100 U/ml], streptomycin [100 μg/ml], and glutamine [2mM] [control medium] or the above medium containing 50 μg/ml L-azetidine carboxylic acid [LACA] or *cis*-hydroxyproline [*cis*-HPRO] [Sigma Chemical Co., St. Louis, Mo]) and incubated as before. The medium was changed every 2 days. In certain experiments the cut coverslips were incubated in medium containing LACA (or *cis*-HPRO) for 2 days, followed by control medium for 2 days, or control medium for the first 2 days, followed by medium containing LACA (or *cis*-HPRO) for the next 2 days. In addition, in some experiments cultures treated with LACA (or *cis*-HPRO) and control cultures were grown in the presence of 200 μg/ml L-proline. Inhibitor-treated cultures showed no evidence of cell death, and migration or migration/proliferation resumed after removal of the inhibitor or by addition of L-proline.

At varying time intervals the coverslips were stained after decanting the culture medium by covering them with Paragon Frozen Section Stain (Paragon C.S.C. Co., Inc., Bronx, NY) for 2 minutes.[9] After washing off the excess stain with water, we mounted the coverslips in water facedown on glass slides. We measured migration, using a calibrated ocular micrometer, by recording the maximal linear distance of cell movement from the cut edge.[9]

In order to address the possibility that irradiation could alter matrix molecule production and expression, we placed confluent cultures in Petri dishes, and cut half the Formvar sheet away. The cultures were analyzed for rate of migration and matrix expression at 1, 2, 3, and 4 days after wounding. Since the area covered by these endothelial cells after "injury" is dependent upon migration and proliferation, the area covered is greater (4000 μ versus 2000 μ observed for irradiated cultures). However, the process is linear, and the matrix components studied did not change in apparent relative amounts or localization when compared with irradiated cultures, as determined by immunofluorescence (data not shown).

Immunofluorescence Staining

Antibodies to collagen Types I, III, IV, and V(AB_2) and laminin were prepared and characterized as previously described.[11-13] Staining of coverslips was accomplished according to previously published procedures.[10] Controls included incubation with secondary antibodies and with inhibited primary antibodies.

We used a Ziess Standard Binocular 14 fluorescence microscope, equipped with a mercury lamp and vertical illuminator with 450- to 490-nm excitation and 520-nm barrier filters to view the slides. Photographs were taken with a 4-minute exposure time with Kodak high-speed Ektachrome film (ASA 400).[11]

Results

Migration of mechanically wounded confluent irradiated calf aortic endothelial cells on coverslips is

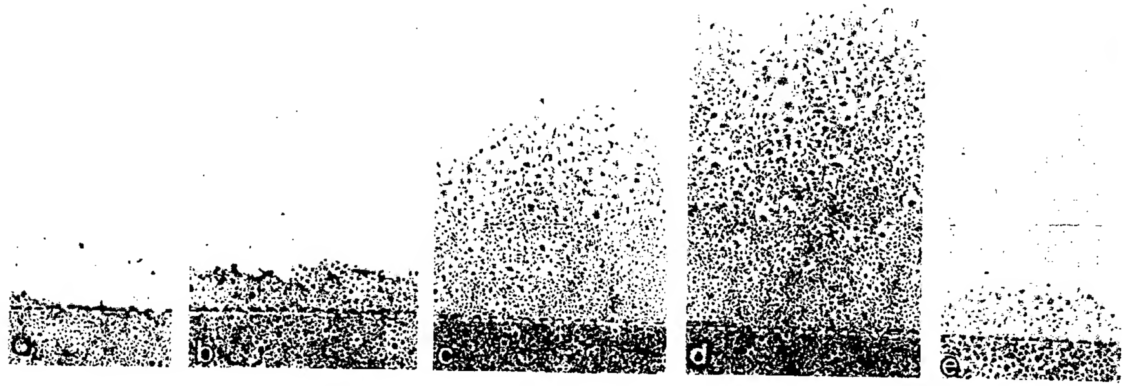

Figure 1—Photomicrographs of migrating endothelial cell cultures that have been "wounded" and incubated in the presence or absence of LACA or *cis*-HPRO. The cut edge is identifiable by a white and black broken line. Endothelial sheet outgrowth in control medium is shown at a) Day 0, b) Day 1, c) Day 3, and d) Day 4. Sheet outgrowth in medium containing LACA (50 μg/ml) is shown at e) Day 4. (× 40)

demonstrated in Figure 1. The scarlet red Formvar coat served as a sharply defined reference front throughout the experiment. (The front is outlined by alternating black and white dashes in Figure 1). The endothelial cells that migrated out from the cut edge of the Formvar did so as a confluent, organized sheet with a relatively smooth, even front (Figure 1A–D). Incubation in the presence of LACA or *cis*-HPRO containing medium significantly inhibited migration (Figure 1E).

The movement of confluent irradiated calf aortic endothelial cells over a 4-day period is recorded in Figure 2. The migration rate was linear during this time period. When coverslips of migrating cells incubated for 2 days in control medium were incubated in medium containing LACA or *cis*-HPRO complete

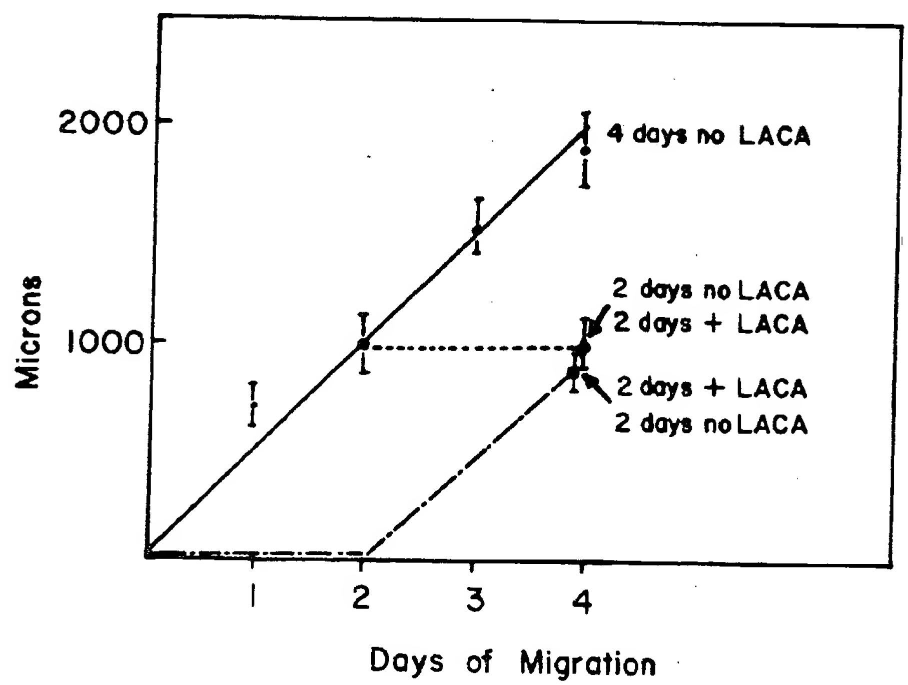

Figure 2—Migration of irradiated, confluent cultures of bovine calf aortic endothelial cells (BAECs). *Solid line*, control cultures measured at 1, 2, 3, and 4 days of migration. *Broken line*, migrating cultures incubated for 2 days in control medium, then inhibited by the addition of 50 μg/ml LACA or *cis*-HPRO for 2 days. *Dotted/broken line*, cultures inhibited for 2 days by 50 μg/ml LACA or *cis*-HPRO, then washed and incubated with control medium for 2 days. All points represent averages of quadruplicate samples. *Bars* represent standard deviations.

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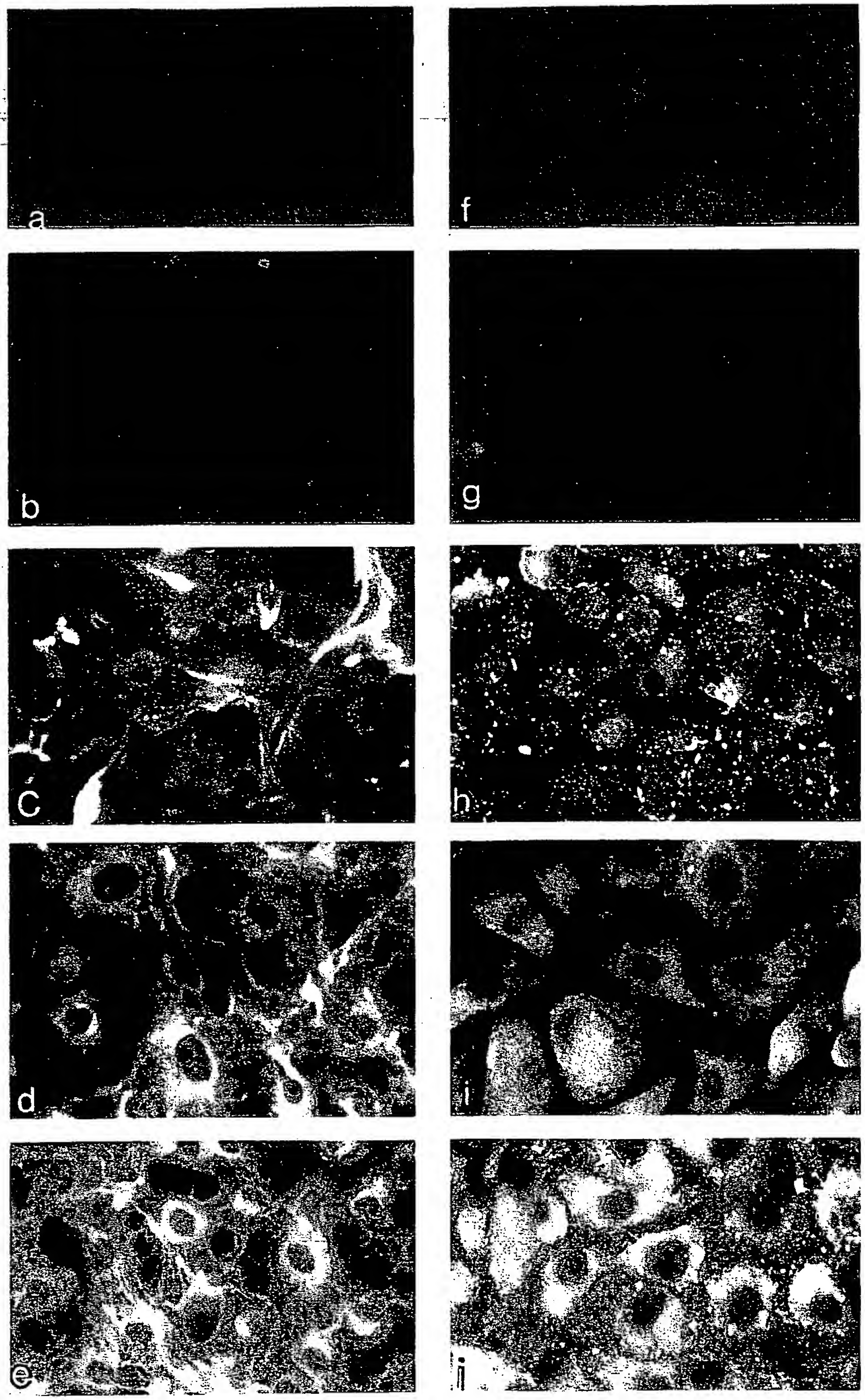

Figure 3—Indirect immunofluorescence patterns of nonmigrating confluent cultures of BAECs stained with antibodies to **a)** Type I collagen, **b)** Type III collagen, **c)** Type IV collagen, **d)** Type V collagen, and **e)** laminin. Note the absence of staining for Types I and III collagen, the lacy reticular staining of the matrix for Type IV collagen, the uniform matrix and cytoplasmic staining for Type V collagen, and the similar pattern for laminin. Indirect immunofluorescence patterns of migrating cultures of BAECs (4 days following "wounding") stained with antibodies to **f)** Type I collagen, **g)** Type III collagen, **h)** Type IV collagen, **i)** Type V collagen, **j)** laminin. Note the absence of staining for Types I and III collagen, the absence of matrix staining for Type IV collagen but the punctate cell-associated staining for this antigen, the uniform cytoplasmic staining for Type V collagen, and the uniform cytoplasmic and punctate cell-surface staining for laminin. (×200)

inhibition of migration was noted at an inhibitor concentration of 50 μg/ml. On the other hand, when coverslips incubated for 2 days in medium containing LACA or *cis*-HPRO were incubated in control medium or medium containing inhibitor to which was added 200 μg/ml L-proline, migration resumed at a linear rate comparable to that of uninhibited cultures.

Since the above experiments suggested that migration and collagen metabolism may be associated, we examined the composition of the matrix molecules produced by migrating and stationary endothelial cells using antibody probes to the various matrix components (Figure 3).

In confluent, nonmigrating cultures no detectable Type I or Type III collagen antigen could be demonstrated, although Type IV collagen was present in a lacy, reticular pattern throughout the matrix. Type V was present in a uniform pattern overlying cell cytoplasm as well as in the matrix, and laminin was observed in a uniform cytoplasmic pattern outlining cells as well as in a delicate, lacy matrix pattern. The collagen distribution pattern found in these endothelial cell cultures was consistent with our previously published data.[10] Migrating endothelial cells 4 days after *in vitro* "wounding" (areas past the Formvar front) also did not stain for Types I and III collagen antigen. Type IV collagen was present, but apparently in reduced amounts, only in a punctate pattern on cytoplasmic surfaces. Type V was present in a uniform intense pattern throughout the cytoplasm of migrating cells, and laminin was present uniformly throughout the cytoplasm with an overlying punctate pattern on cytoplasmic surfaces (Figure 3).

In contrast to the 4-day cultures, when migrating cultures were labeled 1 day after *in vitro* "wounding," the staining patterns were different in that Type V was the only collagen found to be labeled in a uniform cytoplasmic pattern, while Type IV collagen was negative and laminin was weakly positive. At 2 days after "wounding," the cultures stained for Type V collagen in a uniform cytoplasmic pattern and for laminin in a uniform cytoplasmic and punctate pattern cell surface. At this time only weak Type IV labeling was observed in a punctate pattern. When nonirradiated migrating cultures were allowed to achieve confluency, the staining patterns were indistinguishable from those of nonmigrating confluent cultures (data not shown).

Table 1 summarizes the immunofluorescence staining data on nonmigrating and migrating calf aortic endothelial cells in culture under these conditions.

Discussion

Endothelial cell migration is a major early event observed in angiogenesis and in the maintenance of the intact, nonthrombogenic surface of large vessels. Cell migration is a complex phenomenon influenced by many factors, one of which is the substratum. In light of the close physical association between the endothelium and its underlying matrix, it is conceivable that the matrix may in some way(s) influence the migration of endothelial cells. There is already considerable evidence for matrix requirements in epidermal sheet migration as well as for adhesion and spreading of various epithelial and mesenchymal cell types.[14-17] In those studies it was found that epithelial cells preferentially adhered to basement-membrane-associated collagens and appeared to require continual collagen synthesis for migration. In addition, qualitative differences in the types of collagen expressed were noted in the migrating cells.[16] These varied and widespread matrix requirements of epithelial and mesenchymal cell adhesion, spreading, and migration prompted the present study. Albeit, recently, Folkman and Haudenschild found that a

Table 1—Matrix Molecule Localizations in Migrating and Stationary Bovine Aortic Endothelial Cell Cultures (BAECs)

	Collagen type*				
Cell type/culture conditions	I	III	IV	V	Laminin
BAECs—nonmigrating, confluent	—	—	Matrix labeling only	Uniform cytoplasmic and matrix labeling	Uniform cytoplasmic and matrix labeling
BAECs—migrating Day 1	—	—	—	Uniform cytoplasmic labeling	Weak cytoplasmic and punctate labeling
BAECs—migrating Day 2	—	—	Weak punctate labeling	Uniform cytoplasmic labeling	Uniform cytoplasmic and punctate labeling
BAECs—migrating Day 4	—	—	Punctate labeling	Uniform cytoplasmic labeling only	Uniform cytoplasmic and punctate labeling

gelatin matrix was necessary for the successful culture and migration of capillary endothelial cells.[8]

In these studies the linear migration of cultured bovine aortic endothelial cells (BAEC) was reversibly inhibited by agents that are thought to hamper collagen secretion (LACA and *cis*-HPRO), allegedly by proline substitution and formation of abnormal collagens, which do not form the usual triple helical conformation.[17] These observations support the notion that BAECs require continual collagen secretion for migration. Although it is widely accepted that these proline analogs have their effects by altering collagen secretion, the possibility that both these analogues could block cell motility by a non-specific reversible toxic effect is not excluded by these experiments even though the inhibitory effect was reversible in the presence of high concentrations of L-proline.[18-23] The inhibition experiments, in addition to the finding that Type V (AB_2) collagen is the predominant collagen antigen in the migrating cells, are in accord with our previous studies of murine epidermal sheet migration.[16] The localization of this collagen type in capillary basement membranes[11,12] and in the subendothelium of large vessels[10,24] is consistent with its potential role as an early-synthesized component of the matrix upon which cells migrate. The later appearance of Type IV and laminin (other components of basement membranes) in matrix localizations is consistent with this notion. This staggered appearance of matrix components associated with migrating cells is also noted in epiboly experiments using human skin explants.[25] In that system the earliest deposited basement membrane component was bullous pemphigoid antigen followed by laminin and then Type IV collagen. Type V collagen was not assayed for in that study. The reason(s) for this apparently ordered appearance of matrix components during and following the migratory response is as yet unknown. One previously suggested possibility is that specific molecules are necessary for the initial adhesion and migration of cells and that once the cells have covered an area and ceased moving, they have other requirements that call for the synthesis of other basement membrane components, leading to the production of a stable, morphologically identifiable basement membrane.[25]

If this notion is correct, the composition of the remaining underlying substratum following injury may be an important factor in determining the rate and extent of reendothelialization. Since different matrix components are localized to different levels of the vessel wall (collagen Types IV and V to the subendothelium and collagen Types I and III to the media[10,18], the depth of injury may be a factor in determining the endothelial cell migration rate. If, after desquamation, the subendothelium is intact, migration may proceed at a maximal rate, because the matrix components associated with the migratory response are present. However, if the injury is more severe and the subendothelium is destroyed, the subsequent endothelial cell migration would be slowed by the requirement to synthesize the basement membrane components necessary for migration.

Thus, in conclusion, our *in vitro* migration assay system may be of help in further elucidating the matrix requirements of large vessel endothelial cell migration following injury as well as for investigating the dynamics of basement membrane formation and organization following injury. Furthermore, the findings that proline analogs block endothelial cell migration and that the deposition of matrix antigens appears in a sequential order from stationary to motile cells suggest that matrix molecule biosynthesis (secretion) and remodeling are central to the process of endothelial cell motility.

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